BIOTIGER™, A NATURAL MICROBIAL PRODUCT FOR ENHANCED HYDROCARBON RECOVERY FROM OIL SANDS.

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ABSTRACT

BioTiger™ is a unique microbial consortia that resulted from over 8 years of extensive microbiology screening and characterization of samples collected from a century-old Polish waste lagoon. BioTiger™ shows rapid and complete degradation of aliphatic and aromatic hydrocarbons, produces novel surfactants, is tolerant of both chemical and metal toxicity and shows good activity at temperature and pH extremes. Although originally developed and used by the U.S. Department of Energy for bioremediation of oil-contaminated soils, recent efforts have proven that BioTiger™ can also be used to increase hydrocarbon recovery from oil sands. This enhanced *ex situ* oil recovery process utilizes BioTiger™ to optimize bitumen separation. A floatation test protocol with oil sands from Ft. McMurray, Canada was used for the BioTiger™ evaluation. A comparison of hot water extraction/floatation test of the oil sands performed with BioTiger™ demonstrated a 50% improvement in separation as measured by gravimetric analysis in 4 h and a five-fold increase at 25 hr. Since BioTiger™ performs well at high temperatures and process engineering can enhance and sustain metabolic activity, it can be applied to enhance recovery of hydrocarbons from oil sands or other complex recalcitrant matrices.

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Oil Sands

Recovering oil from the Athabasca Oil Sands has been a major technological challenge. While many methods have been successful, the fundamental mechanisms of hydrocarbon extraction from oil sands are not straightforward. The heterogeneity of the oil sands including fines content, bitumen composition, clay mineralogy, and other factors can influence the extraction process. Current methods employ multi step systems of heating, aqueous phase separation, mechanical mixing, aeration, and chemical additions to extract hydrocarbons from the oil sands. Past efforts have generated large tailings ponds that still contain varying amounts of bitumen indicating the poor efficiency in that process. Recent environmental concerns have included the amount of water used in the process, energy cost to operate the systems, runoff from the tailings ponds, wastewater from the facilities, as well as chemical residues (e.g. paraffins) in the water left over from the

extraction process. Ironically, while the value of the oil sand increases, so does the energy costs it takes to recover the material. With the increasing value of petroleum, more product recovered per unit volume oil sand would be desirable. In addition methods to upgrade the separated hydrocarbons would be advantageous for shipping, transportation, and downstream processing. New technology is also need for oil sands that are too shallow for current cost-effective *in situ* methods or present at undesirable depths. Lower cost and environmentally compatible methods to leverage existing technologies is an optimal solution.

Microbiology of Oil Sands

The microbiology of petroleum began in the 1800's when Russian scientists first investigated "souring" of oil in subsurface reserves. In recent years microbiologists and engineers have tested bacteria as a means of improving oil recovery through various means including plugging sediment pores to create more efficient collection techniques and harnessing microbial gas production to enhance pumping efficiency. Oils sands have a unique microbiological signature that varies with geographical location. Techniques for the characterization of potential microbial activity within the Athabasca oil sands formation have indicated significant seasonal changes. However, hydrocarbon biodegradation of the oil sand-adapted mixed sediment population has not correlated well with the concentrations of bituminous hydrocarbons in specific site sediments. These results suggest that a general capability for hydrocarbon oxidation exists in the Athabasca River system and that this capability is enhanced within the natural bounds of the Athabasca oil sands. The bacteria within these oil sands can metabolize available hydrocarbons for growth. The oil sands naturally contain high concetrations of polyaromatic hydrocarons (PAHs) including haphthenic acids. These naphthenic acids are a complex family of cyclic and acyclic carboxylic acids that are present in the acidic hydrocarbon fraction of oil sands. These PAHs, including naphthenic acids, are of concern as they are acutely toxic to aquatic organisms and some are carcinogenic. Previous studies showed that downstream wetland sediments exposed to oil sands process water containing naphthenic acids had higher rates of naphthenic acid biodegradation *in vitro* compared with similar unexposed areas indicating adaptation. Microbial ecological studies have demonstrated that naphthenic acids-exposed bacterial communities were homogeneous on a scale of meters, whereas unexposed (off-site) areas including wetlands are less homogeneous.

BioTiger™

BioTiger™ is a unique set of bacteria isolated from acidic oil refinery sludge. Initially aerobic microorganisms were isolated from the aged acidic sludge (pH 3) composed of asphaltics highly contaminated with PAHs. The consistency of the sludge was comparable to tar mixed with gravel. Hundreds of bacteria, fungi, and yeast spp. were isolated from the sludge on an acidic minimal agar medium exposed to naphthalene vapor or phenanthrene crystals. A subset of the microbial isolates that grew consistently

on naphthalene as a sole carbon and energy source was characterized by BIOLOG© and analysis of 16S rRNA genes. A number of bacterial isolates grouped within the Proteobacteria and the rRNA gene sequences were similar to *Ralstonia*, *Pseudomonas*, *Sphingomonas*, *Burkholderia*, *Stenotrophomonas*, and *Achromobacter* spp. Some bacterial strains expressed dihydroxylating dioxygenase activity and were able to grow on catechol as a sole carbon source. A bacterial isolate with sequence similarity to *Ralstonia* KN1 was able to grow on naphthalene and degrade phenanthrene. The predominant phenanthrene degraders were related to *Sphingomonas*, *Pseudomonas* or *Burkholderia* spp. One *Sphingomonas* sp. also was able to degrade pyrene and fluoranthene. The results indicated that the diversity of acid-tolerant PAH-degrading microorganisms in acidic oil wastes was greater than previously recognized. BioTiger™ is composed of 12 isolates from that project (Table 1).

Table 1. Isolate identification.

Isolate	Identification
1) CZOR-L1B (KN-1)	*ALCALIGENES-PIECHAUDII SRS*
2) BP-20 (KN-2)	*RALSTONIA PICKETTII SRS.*
3) CZOR-L1Bsm (KN-3)	*PSEUDOMONAS-PUTIDA* BIOTYPE B *SRS*
4) BPB	*FLEXIBACTER CF. SANCTI SRS*
5) BPC	*PSEUDOMONAS FREDRIKSBERGENSIS SRS*
6) BPE	*STAPHYLOCOCCUS WARNERI. LMG 19417 SRS*
7) BPF	*SPHINGOMONAS SRS*
8) BPH	*SPHINGOMONAS SP. S37 SRS*
9) BPI	*PHYLOBACTERIUM SRS* (α PROTEOBACTERIUM TA-A1)
10) BPJ	*SERRATIA FICARIA SRS* (α PROTEOBACTERIUM TA12-21)
11) BPK	*AGROBACTERIUM TUMEFACIENS SRS*
12) BPL	*RHIZOBIUM SP. SDW045 SRS*

Biosurfactant production

The bioavailability of target compounds and microbial nutrients is an important factor in microbial activities including bioremediation. The bioavailability of a chemical may be described by its mass transfer rate relative to its uptake and degradation rates by microorganisms. If the capacity for hydrocarbon degradation is present and environmental conditions are amenable, the microorganisms must have access to the contaminants for degradation. Reduced bioavailability could be caused by low aqueous solubility and strong sorption to soils or sediments. Bioavailability is often an issue with hydrophobic hydrocarbons, particularly PAHs, as the water-dissolved fraction of chemicals is more available to soil microorganisms. The use of surfactants has been shown to increase biodegradation of hydrocarbon contaminants by increasing bioavailability.

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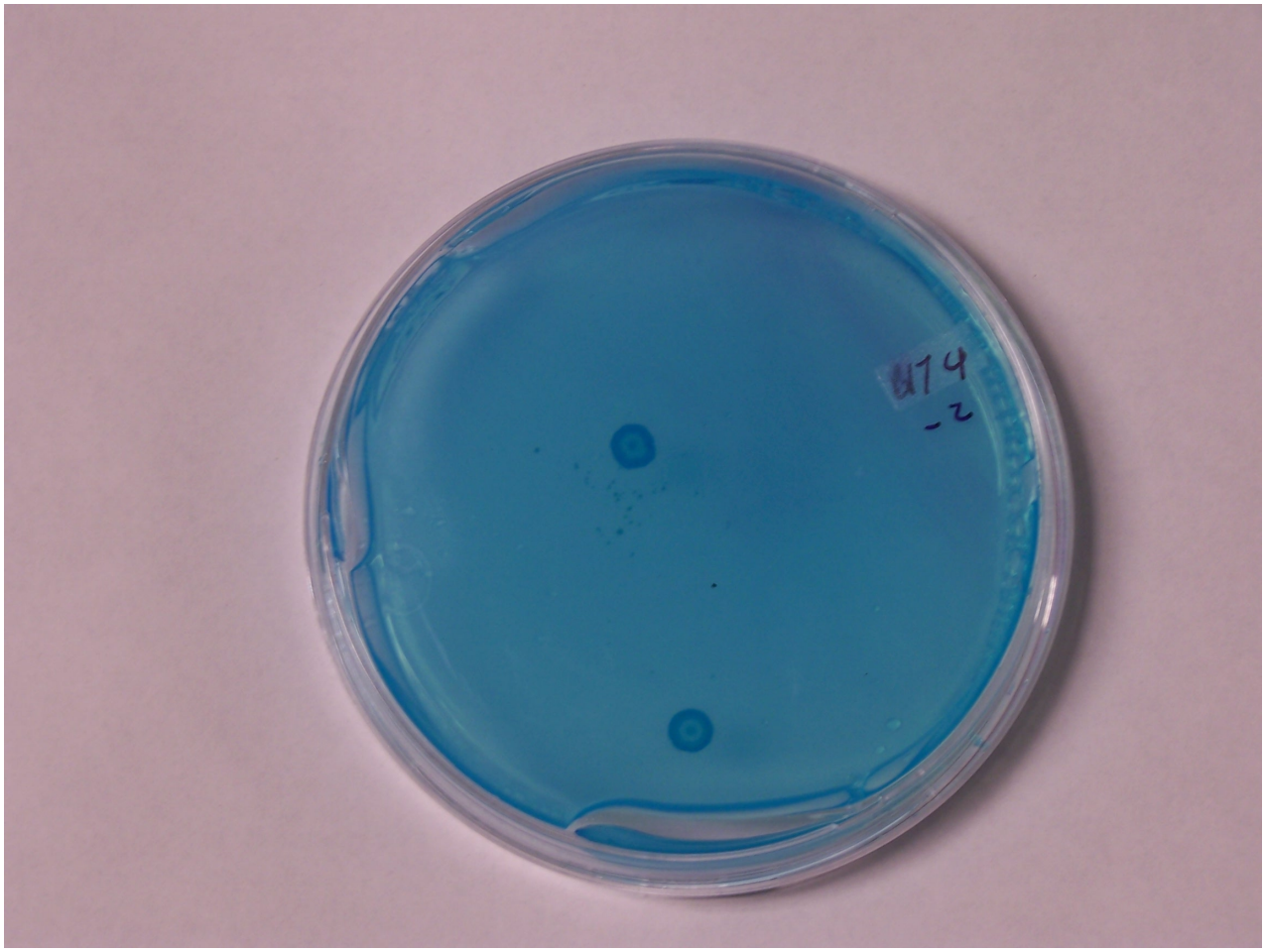
Modifying soil composition and structure through mechanical means or amendments can significantly influence bioremediation activities. Bulking agents are

materials of low density that lower soil bulk density, increase porosity, moisture retention and oxygen diffusion, and can help to form water-stable aggregates increasing aeration and microbial activity. The addition of surfactant-producing non-indigenous microbes or synthetic surfactants has been used in soil treatment to help increase availability of these recalcitrant materials. Moreover, the production and presence of biosurfactants has been shown to have many of the benefits of synthetic surfactants as well as being biodegradable and nontoxic. Several BioTiger™ cultures indicated potential biosurfactant production by producing foam when growing with hydrocarbons. Ionic biosurfactants were evaluated by the methylene blue method. Methylene blue active substances including the biosurfactant rhamnolipids form an ion pair with methylene blue reagent and then this complex can be screened in culture. Figure 1 shows biosurfactant production by the BioTiger™ component CZOR-L1B (KN-*Alcaligenes-piechaudii SRS* after incubation with Athlabasca Oil Sands.

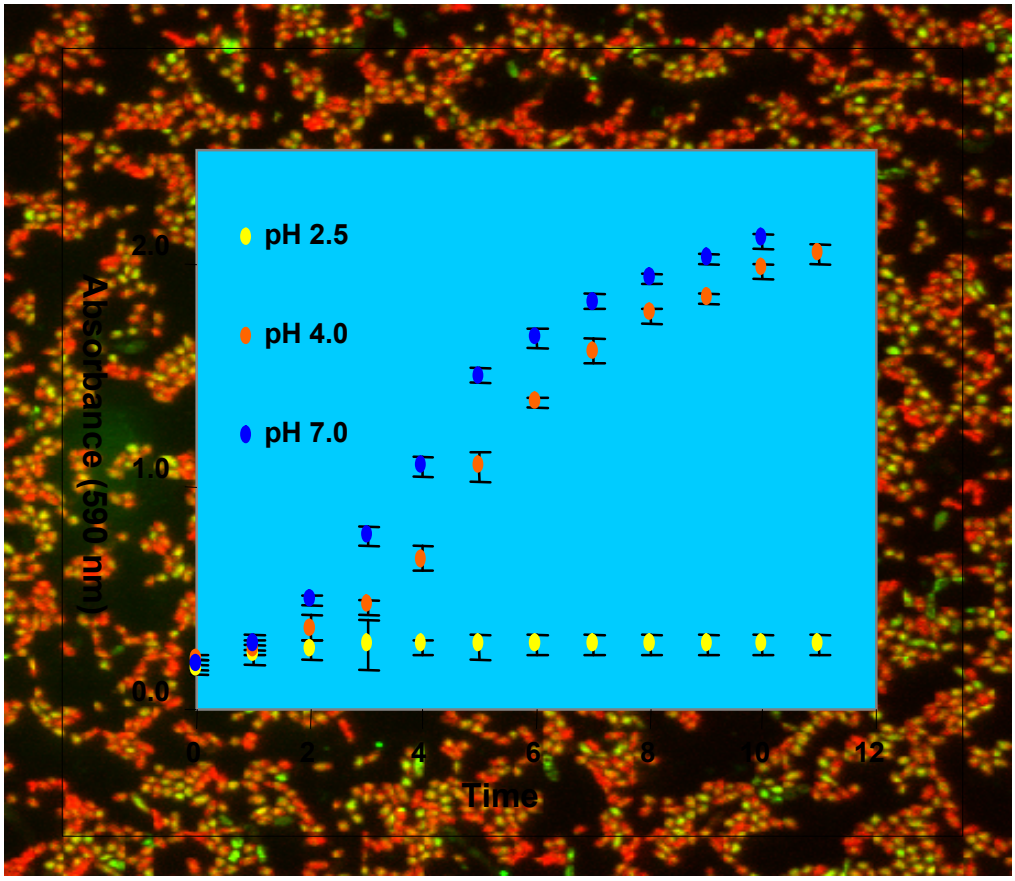
Figure 1. Biosurfactant production by CZOR-L1B (KN1) -*Alcaligenes-piechaudii SRS* as demonstrated by dark blue complex by colonies.



pH

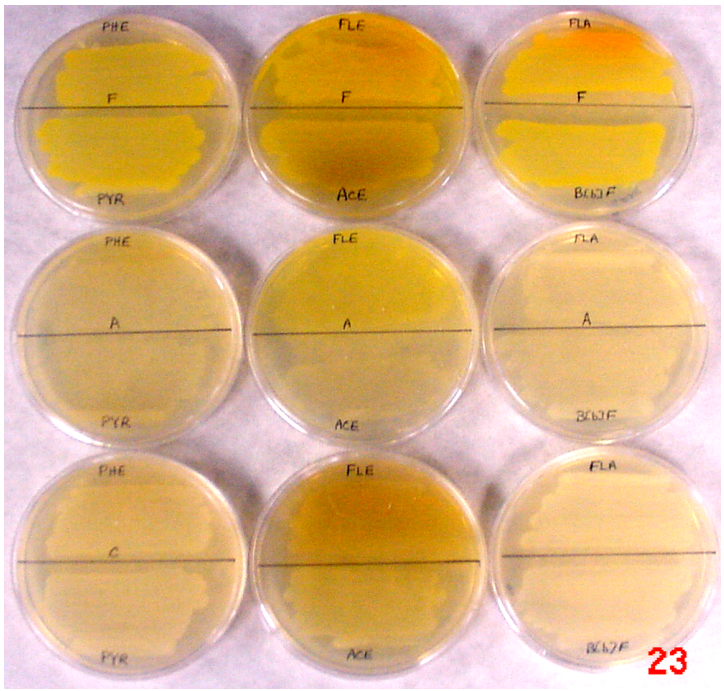
The diversity and catabolic potential of microorganisms isolated from an acidic sludge composed primarily of mixed PAH and heavy metals was investigated. The goal of this study was to investigate the diversity and catabolic potential of microorganisms existing in an acidic sludge lagoon from oil refinery waste. The Czechowice oil refinery in Poland had a long history of high level PAH and heavy metals exposure (100 years) and is acidic (pH 3). Investigations of this site involved identifying factors that stimulate microbial degradation of petroleum contaminants and an enhanced bioremediation system has been implemented for an emptied oil waste basin. Most previous studies of have focused on PAH degrading microbial isolates obtained from environments that are not considered extreme. The presence of acidophilic microorganisms has been documented in acidic PAH contaminated environments. A few studies have demonstrated PAH degradation at low pH and degradation by heterotrophic acidophiles. Figure 2 demonstrates growth of BioTiger™ in acidic conditions (pH 4).

Figure 2. Growth of BioTiger™ in acidic (pH 4) conditions



Catabolic potential of BioTiger™

Naphthalene, anthracene, phenanthrene, pyrene, acenaphthene. fluorene, fluoranthene, catechol, indole, 2,3- hydroxynaphthoic acid, 1,2 dihydroxynaphthalene, naphthenol, 9-fluorenone, 9-hydroxyfluorene, acenaphthol, 2,3- and 3,4 dihydroxybenzoic acid PAHs and standards were dissolved in acetone and filter sterilized. After verification of clearance of phenanthrene crystals by isolates on agar plates sprayed with a solution of hexane saturated with phenanthrene, the isolates were check for purity. Purified isolates demonstrating clearance of phenanthrene crystals were then tested with the following compounds as potential degradation substrates; catechol, indole, anthracene, fluorene, acenaphthene, fluoranthene, and pyrene. Catechol 2, 3 dioxygenase activity was determined by spraying a 0.1% catechol solution onto isolated colonies from cultures grown on a glucose (1%) minimal agar medium after incubation of 24 - 48 h. A positive reaction was observed as the appearance of a yellow color surrounding the colonies with 5 min indicating *meta* ring cleavage of catechol. Dihydroxylating dioxygenase activity was determined with pure cultures growing on medium containing 1mM indole. A positive reaction was observed as the appearance of blue colonies indicating indigo production. Saturated solutions of each PAH were prepared in acetone. The PAH substrate range was tested by adding a streak of the acetone dissolved PAH to one edge of an agar plate using a sterile cotton swab and adjacent to a confluent streak of the microbial isolate. Degradation of the PAH was indicated by the disappearance of PAH crystals and/or appearance of chromogenic degradation products diffusing into the agar medium after incubation. (Figure 3)



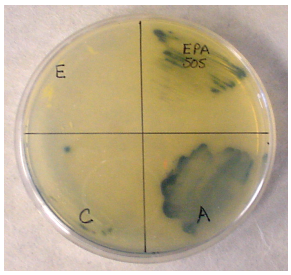


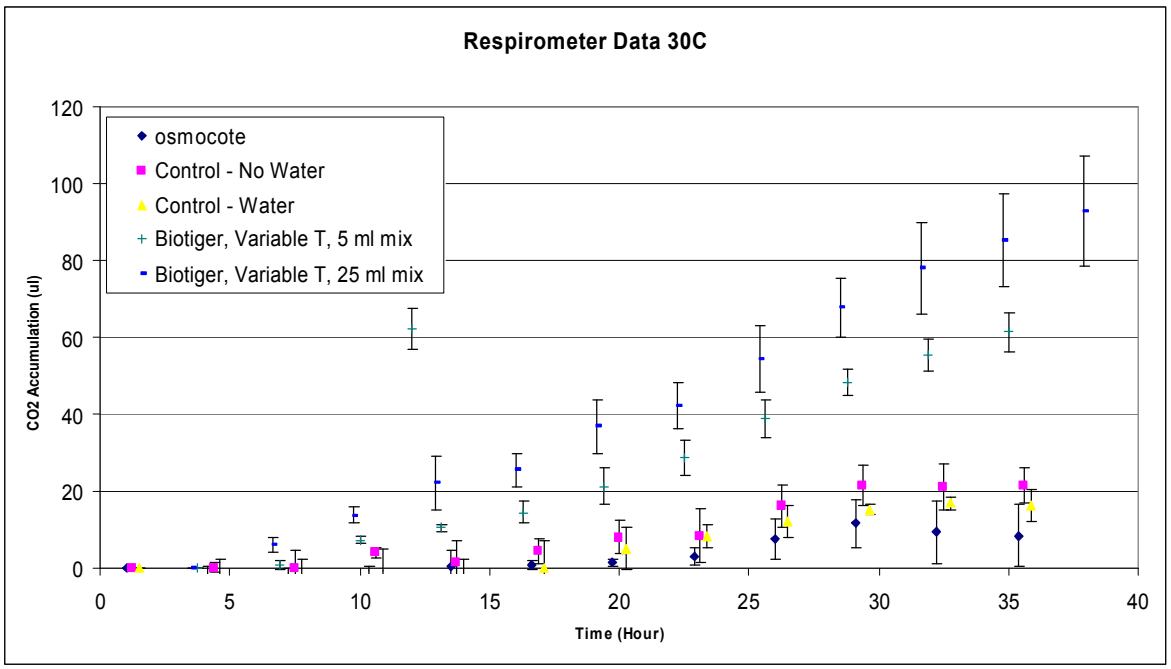
Figure 3. Representative bacterial isolates that demonstrate a positive response in conversion of color gradients from tan to indigo and/or degradation of phenanthrene (PHE), pyrene (PYR), fluorene (FLE), acenaphthene (ACE), fluoranthene (FLA), and benzo[b]fluoranthene (B[b]F).

Temperature

Temperature is an important parameter for most bioremediation sites because of its impact on the availability of contaminants and the activity of the microorganisms. Especially true in northern latitudes, seasonal variation can also impact bioremediation sites. For optimal contaminant removal, biological treatment of organic pollutants such as petroleum-based hydrocarbons is performed at moderate temperatures (20° to 37°C) in order to increase metabolic activity, diffusion, and mass transfer

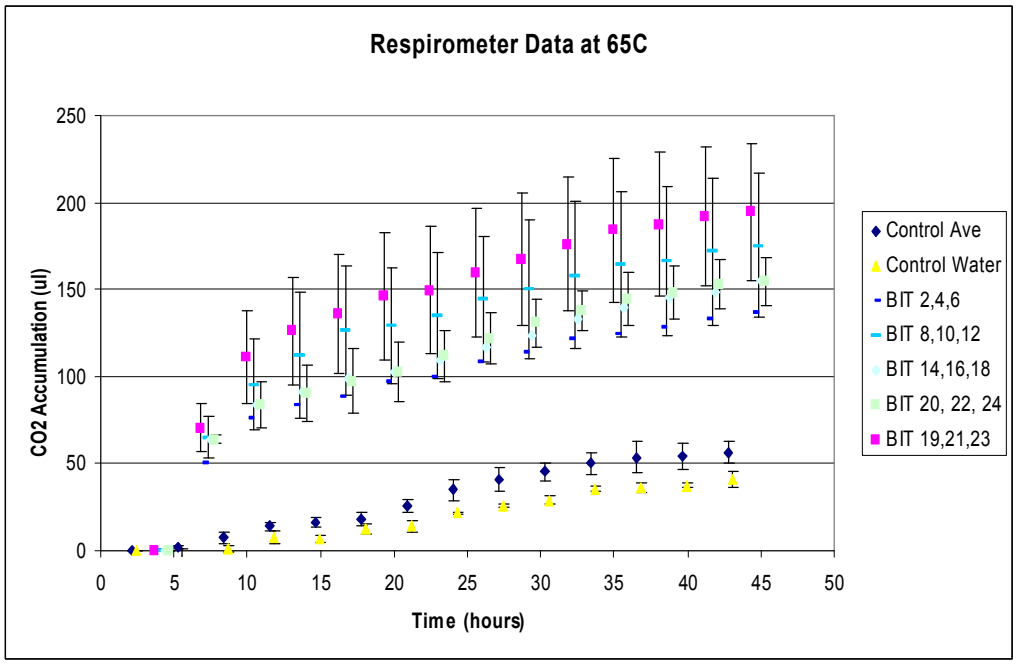
Costs and environmental impact can be significantly reduced if energy requirement are minimized and/or solvent-bitumen can be reduced. While in the past reducing the temperature or the solvent-bitumen ratio would lead to lower bitumen recovery or quality. Therefore, it would be advantageous to use a biologically enhanced process under process conditions that would optimize bitumen recovery and hydrocarbon quality while reducing temperature/energy requirements and bitumen-solvent applications. Experiments were set up with a respirometer to measure microbial metabolism including O_2 consumption and CO_2 production. A shaking water bath was employed to control temperature and mixing. Initially BioTiger™ was incubated with 10 grams oil sands at two concentrations, a 5 ml and 25 ml inoculum, total volume 100 ml in sterile filtered water, two controls with and without water, and with a commercial fertilizer Osmocote ™ at 30º C. Results showed a high metabolic rate for BioTiger™ with Oil Sands as the sole carbon and energy Source that correlated with microbial concentration as compared to controls (Figure 4A).

Figure 4A. BioTiger™ metabolism with Oil Sands @ two concentrations, two controls, and with Osmocote ™ at 30º C.



A second experiments were set up with a respirometer to only using BioTiger™ that had been incubated with 10 grams Oil Sands with different mineral micronutrients, total volume 100 ml in sterile filtered water, and two controls with and without water at 65º C. The temperature 65º C was selected as this one operating parameter for some of the Ft McMurray oil sand separation systems. Results showed a high metabolic rate for BioTiger™ with Oil Sands as the sole carbon and energy that that again correlated with microbial concentration as compared to controls (Figure 4B). The metabolic rates were higher than @ 30º C (Figure 4A). In this experiment (Figure 4B), BioTiger™ had been 'primed' incubating with oil sands.

Figure 4B. BioTiger™ metabolism with Oil Sands @ five concentrations and two controls @ 65º C.



BioTiger™ activity with Oil Sands: Hydrocarbon Recovery

Figure 5 is a photo taken with a Laser Scanning Confocal Micrograph showing BioTiger™ bacteria attached to oil sand particles. Attachment of the bacteria is no doubt important for separation of oil/hydrocarbons from the sand particles. The process of BioTiger™ attachment to oil sand particles was very rapid and was observed within one hour of incubation. The untreated oil sands were observed to have some indigenous bacteria but densities were very low.

Figure 5. BioTiger™ bacteria attached to oil sand particles.

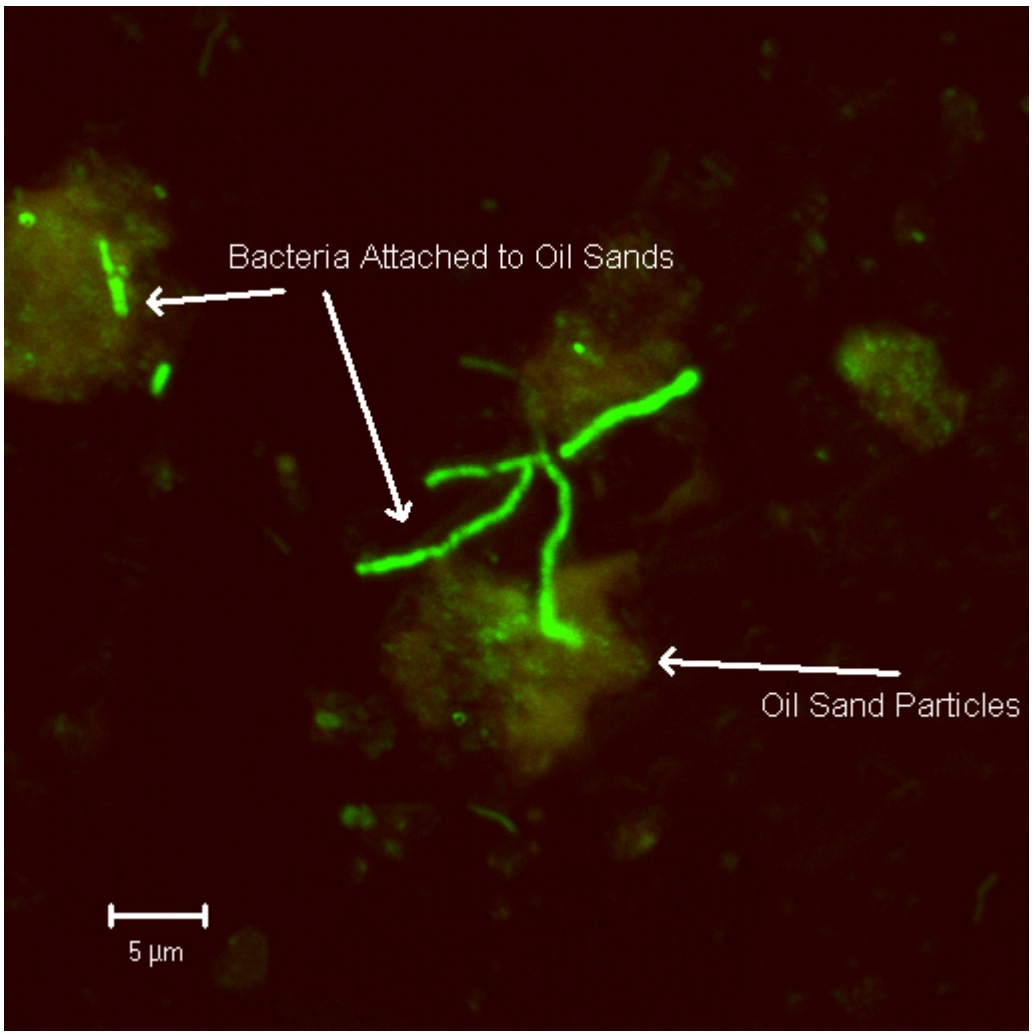
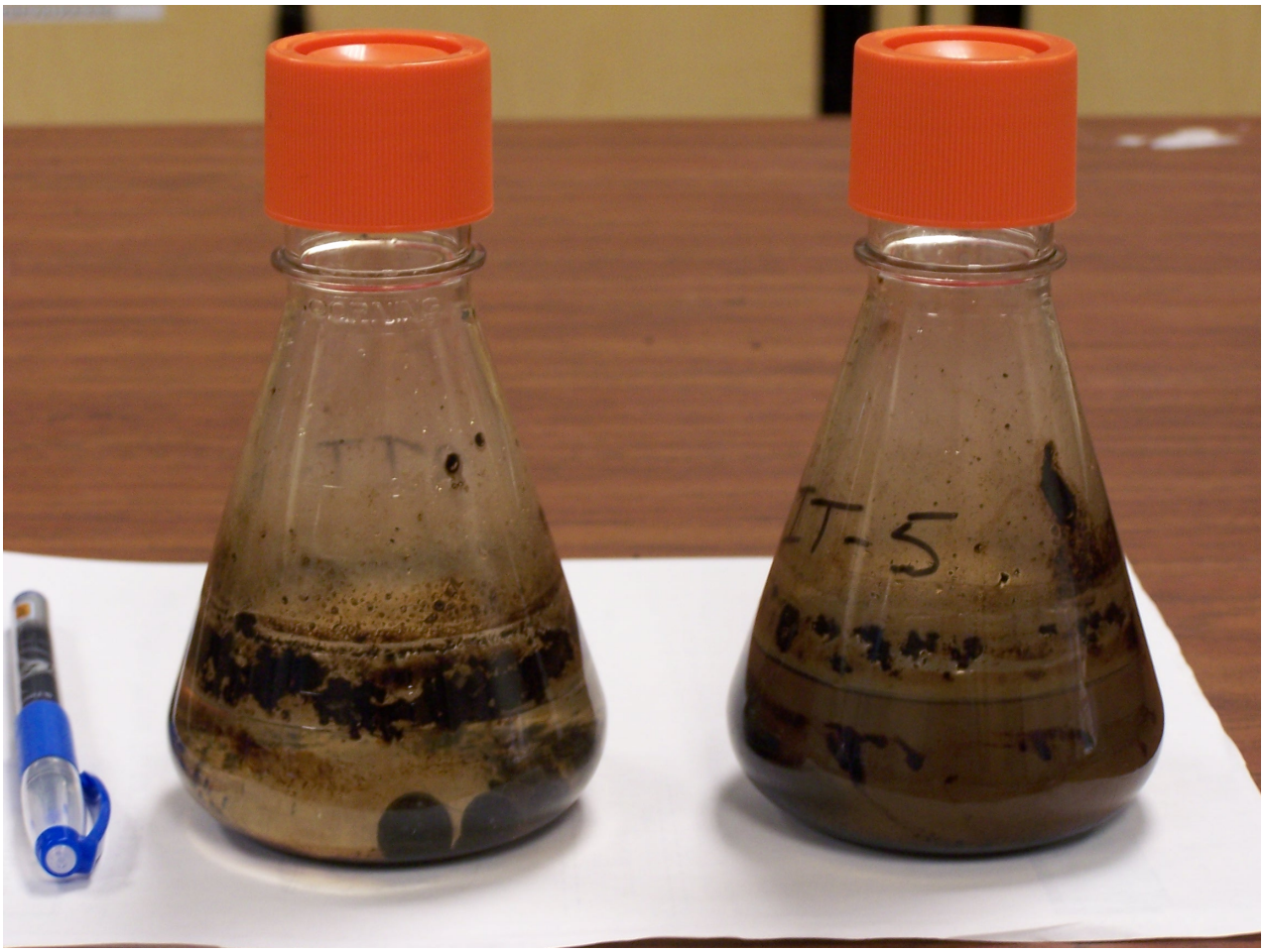


Figure 6 shows BioTiger™ treated and untreated (sterile water only) oil sands in a 250 ml flask after shaking at 25º C for 1 week. This experiment demonstrates better separation with BioTiger™. A layer of sand was clearly evident on the bottom of the BioTiger™ treated flask.

Figure 6. BioTiger™ treated (left) and Untreated Oil Sands (right) in a 250 ml flask after shaking at 25º C for 1 week.

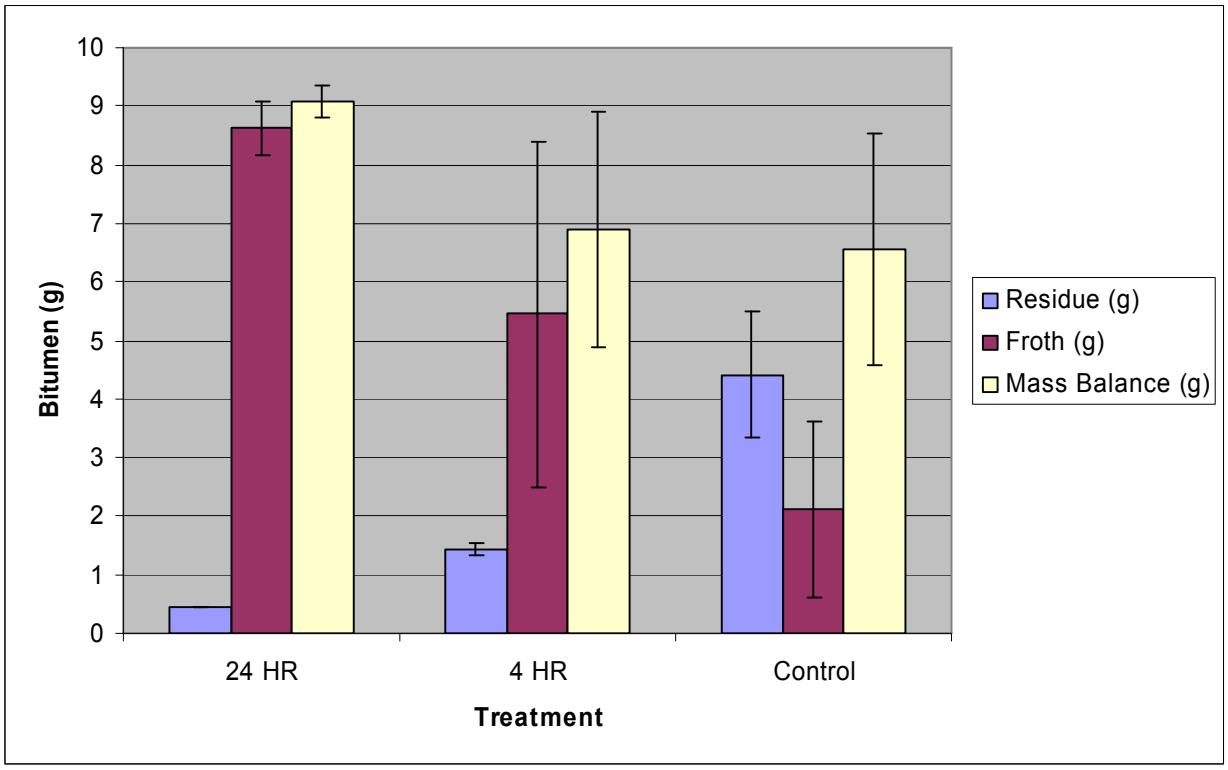


Bitumen Recovery from Oil Sands

Currently a hot water process for extracting bitumen from tar sands requires forming a mixture of tar sands and water, settling the mixture in a primary extraction zone to form an upper bitumen froth layer, a middlings layer, and a sand tailings layer, passing a part of the middlings layer to an air scavenger zone to recover additional bitumen. Improvements to the process include aerating the mixture of tar sands and water before separating bitumen froth from the mixture in a gravity settling zone. This aeration can be accomplished by adding an aerated recycle middlings stream to the tar sands water-mixture prior to the settling step. The middlings stream can still contain from 2 to 6% bitumen. The middling stream contains from 8-24% fines and the balance is water. Figure 7 shows the result of increased bitumen recovered from Athlabasca tarsands with Biotiger at 4 and 34 hours as compared to controls with no Biotiger added. A modified

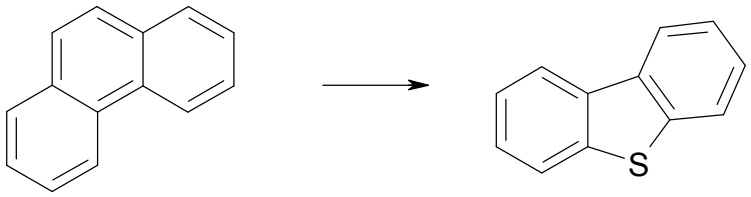
hot water extraction process was used with laboratory glassware and 50 grams of tar sands.

Figure 7. Floatation Test Data (modified)



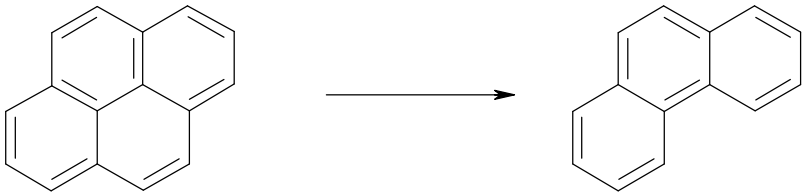
A series of experiments was set up to evaluate BioTiger™ processing of oil sands as well as other hydrocarbons. A number of metabolic products, both organic and inorganic, were observed and analyzed by GC/MS. Some chemical by products of PAHs were observed both in controls and BioTiger™ treated samples. For example, chemical transformations observed both with aqueous controls as well as with BioTiger™ included phenanthrene conversion to dibenzothipene (Figure 7A) . This abiotic conversion was observed within 1 hour as well as after several days on incubation and repeatedly occurred both abiotically with water controls and biotically with BioTiger™. However, pyrene was converted to phenanthrene only when incubated with BioTiger™ (Figure 7B).

Figures 7A. Phenanthrene conversion to dibenzothipene occurs both abiotically and biotically with BioTiger™



Phenanthrene Dibenzothiphene

Figures 7B Pyrene conversion to phenanthrene occurred only biotically with BioTiger™



Pyrene Phenanthrene

Figure 8A demonstrates BioTiger™ cometabolic growth on hydrocarbons ACE, PHE, PY, and nAlkanes (nAlk). Figure 8B demonstrates BP20, one of the BioTiger™ components cometabolic growth on hydrocarbons ACE, PHE, PY, and nAlk. Note the longer lag time for the individual BP20 (Figure 8A) to grow vs the single culture (Figure 8B). This work demonstrates the efficiency of the BioTiger™ consortia to metabolize hydrocarbons.

Figure 8A. BioTiger™ Growth on select hydrocarbons

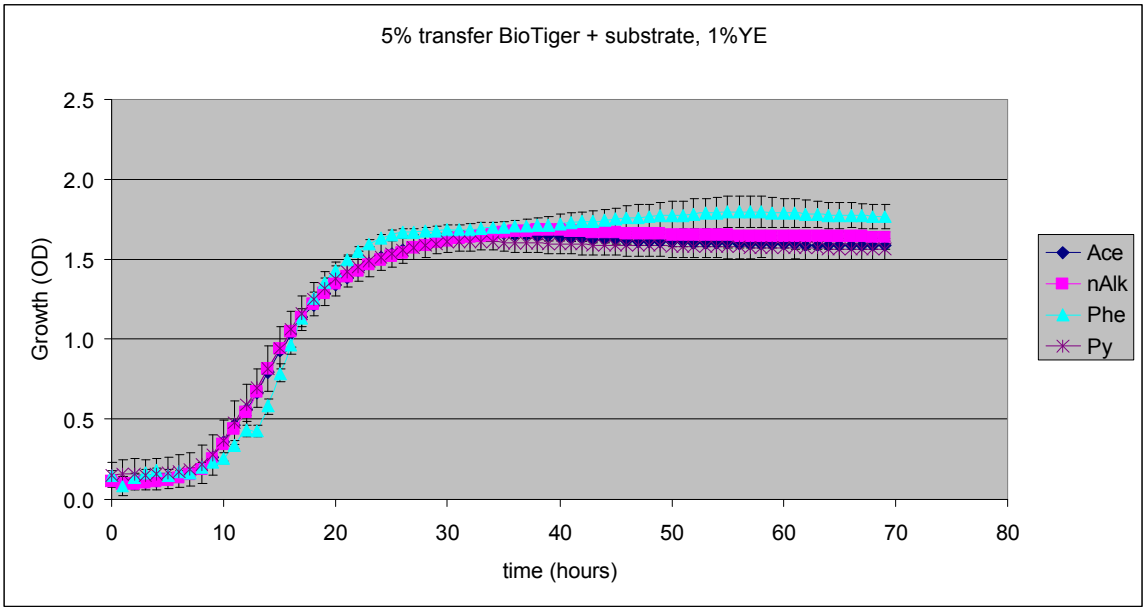
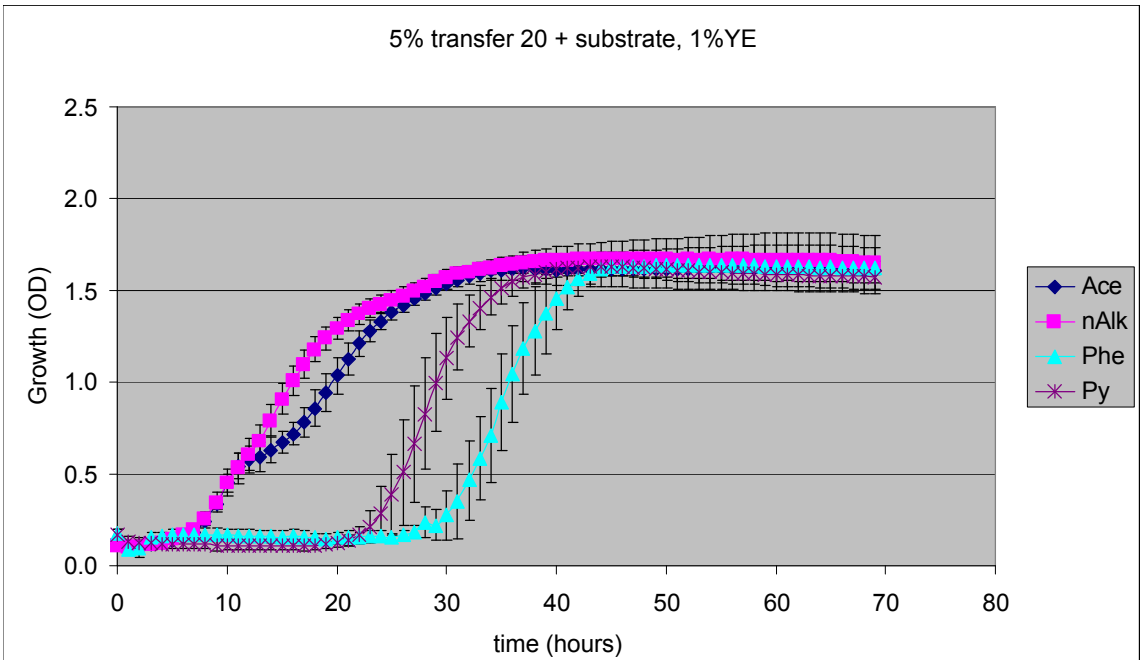


Figure 8B. Individual BioTiger™ bacterium Bp20 growth on select hydrocarbons



Summary

BioTiger™ is made up of twelve bacteria isolates proven to have consistent activity for bioremediation of petroleum compounds. These resilient organisms added for bioaugmentation are listed in Table 1. Isolates 1-3, *Alcaligenes piechaudii* SRS, *Ralstonia pickettii* SRS, and *Pseudomonas-putida* Biotype B SRS, all demonstrate the ability to produce biosurfactants in the presence of petroleum compounds, the formation of which was noted during culturing conditions with petroleum as the sole carbon & energy source (Table 1). All the isolates all demonstrate the ability to biodegrade and metabolize a variety of petroleum hydrocarbons to varying degrees. This unique patent pending microbial consortia has also the proven the ability to enhance hydrocarbon separation from oil sands without added chemicals. In recent years the Canadian Oil Sands Industry has been increasing capacity due to ongoing petroleum supply and demand issues. While new technologies will be tested to maximize those efforts those that can minimize the environmental impact, remain cost effective, while enhancing recovery will be the processes of choice. BioTiger™ conversions of PAHs may be used for upgrading activity to enhance downstream processing. The extent of BioTiger™ applications to oil sands for recovery processes, both *in situ* and *ex situ*, is remained to be fully explored.